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Purification of the Two Major Forms of Rat Pituitary Corticotropin Using Only Reversed-Phase Liquid Chromatography†

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ABSTRACT: Reversed-phase liquid chromatography has been used to extract and purify to homogeneity the two major forms of corticotropin (ACTH) from 60 rat anterior pituitaries. Tissue was homogenized in a medium designed to minimize peptidase activity and maximize solubilization of peptides. The supernatant obtained from the tissue homogenate was extracted in a batch procedure with octadecylsilylsilica (ODS-silica). Elution of the ODS-silica gave rise to a desalted and essentially protein-free preparation, which was enriched in peptides. From this initial extract, the two major forms of rat ACTH were purified to apparent homogeneity by reversed-phase high-performance liquid chromatography (RP-HPLC), using solvent systems containing either trifluoroacetic acid or heptafluorobutyric acid as hydrophobic counterions. The recovery of ACTH immunoreactivity through the tissue extraction and chromatography stages was close to 100%. In control experiments, it was observed that the structural integrity of synthetic tritiated human ACTH was maintained throughout the extraction and purification procedures. The two forms of rat ACTH were found in approximately equimolar amounts, with very similar amino acid compositions which indicated a close similarity to other mammalian corticotropins. Both forms were found to have biological activities and molecular weights comparable to standard synthetic human $ACTH_{1-39}$. Trypsin digestion indicated that the two peptides were identical except for a modification of one form in the carboxyl-terminal tryptic peptide. Initial radiolabeling experiments, using cultured rat anterior pituitary cells, have shown that the more polar form of rat ACTH is O-phosphorylated on the serine residue at position 31.

Reversed-phase high-performance liquid chromatography (RP-HPLC)[1] is proving to be a very useful technique in the preparation of both natural and synthetic polypeptides (Rivier, 1978; Hancock et al., 1978). High resolution of closely related compounds can be achieved with the chromatographic supports which are now available. However, RP-HPLC is almost invariably used in the final purification step which follows more conventional ion-exchange and gel-filtration chromatographic procedures. For instance, such methods have been used to purify several natural peptide hormones, including α-endorphin and γ-endorphin from a rat hypothalamic–hypophyseal extract

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[1] Abbreviations used: RP-HPLC, reversed-phase high-performance liquid chromatography; ACTH, corticotropin; α-MSH, α-melanotropin; β-LPH, β-lipotropin; RIA, radioimmunoassay; $NaDodSO_4$, sodium dodecyl sulfate; F_3CCOOH, trifluoroacetic acid; HFBA, heptafluorobutyric acid.

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(Ling et al., 1976), β-endorphin and β-lipotropin from the rat pituitary (Rubenstein et al., 1977a,b), and somatostatin and related peptides from the porcine intestine (Pradayrol et al., 1978, 1980) and the pigeon pancreas (Speiss et al., 1979). It has recently been shown that reversed-phase liquid chromatography can be used for both the extraction and complete purification of peptides (Bennett et al., 1980a,b). One of the most widely used supports in the RP-HPLC of peptides has been octadecylsilylsilica (ODS-silica) (Burgus & Rivier, 1976; Bennett et al., 1977; Hancock et al., 1978). ODS-silica has a high affinity for peptides and has been used successfully to extract peptides from whole plasma (Bennett et al., 1977) and from tissue homogenates (Bennett et al., 1978).

The objective of the work presented here was to adapt and optimize ODS-silica extraction procedures and use them in combination with RP-HPLC to realize a general method for the rapid and complete purification of peptides with the minimum of losses and artifacts. As a model system to demonstrate the potential of this new methodology, we have chosen to study peptides related to corticotropin (ACTH) in the rat pituitary. The existence of heterogeneous forms of ACTH in various species has long been recognized (Yalow & Berson, 1973). More recently, the pathways for the biosynthesis of ACTH have been intensively investigated in mouse pituitary tumor cells in culture (Mains & Eipper, 1976). These studies have demonstrated the existence of a common precursor to ACTH and β-lipotropin (β-LPH) (Mains et al., 1977), designated 31K from its apparent molecular weight on sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis, intermediate forms of ACTH designated 23K and 13K (Eipper et al., 1976), and the low molecular weight 4.5K form ($ACTH_{1-39}$). An extension of these studies to the rat anterior pituitary revealed that a similar pattern (31K, 20K, 14K, and 4.5K) of ACTH immunoreactivity was present (Eipper & Mains, 1978). In these studies, the various forms of ACTH were identified by a combination of $NaDodSO_4$–polyacrylamide gel electrophoresis and sequence-specific radioimmunoassays. None of these molecules have been isolated from rat pituitaries and positively identified by the classical methods of peptide mapping and amino acid analysis. In the work presented here, we have purified to apparent homogeneity the two major forms of ACTH from the rat anterior pituitary by using only reversed-phase liquid chromatography and have positively identified them by amino acid analysis and peptide mapping.

Experimental Procedures

Materials. Synthetic peptides were generously donated by the following: human calcitonin, α-melanocyte-stimulating hormone (α-MSH), Synacthen ($ACTH_{1-24}$), and human $ACTH_{1-39}$ from Dr. W. Rittel, Ciba-Geigy Ltd., Basel, Switzerland; methionine enkephalin and somatostatin from Dr. R. Degenghi, Ayerst Laboratories, Montreal, Canada; human ACTH fragments 1–18, 16–27, 20–27, and 22–39 from Dr. K. Inouye of Shionogi Co., Osaka, Japan; porcine α-, β-, and γ-endorphins from Dr. R. Guillemin and Dr. N. Ling, Salk Institute, La Jolla, CA; human [3,5-$^3H_2$]Tyr^{23}-$ACTH_{1-39}$ (Brundish & Wade, 1977) from Dr. R. Wade, Ciba Laboratories, Horsham, Sussex, United Kingdom; human $ACTH_{17-39}$ from Dr. P. J. Lowry, St. Bartholemew's Hospital, London United Kingdom. Porcine insulin was obtained from Dr. E. L. Grinnan, Eli Lilly Co., Indianapolis, IN. Porcine $ACTH_{1-39}$ (86 units/mg) was purchased from Sigma Chemical Co., St. Louis, MO. Trifluoroacetic acid (F_3CCOOH, laboratory reagent grade) was obtained from BDH Chemicals Ltd., Montreal, Canada, and heptafluorobutyric acid (HFBA, purissimum grade) from Tridom Chemical Inc., Hauppauge, NY. Acetonitrile (HPLC grade) was purchased from Fisher Scientific, Montreal, Canada.

High-Performance Liquid Chromatography. The Waters HPLC system consisted of two 6000A pumps, a 660 solvent programmer, and a U6K injector (Waters Scientific, Mississauga, Canada). A Waters Associates C_{18} μBondapak column was used for all peptide purification procedures. Column eluates were monitored for UV absorbance by using two flow-through variable wavelength spectrophotometers connected in series (Perkin-Elmer, Montreal, Canada). A Model LC-75 equipped with a 1-cm path length cell measured absorbance at 210 nm while a Model LC-55 equipped with a 0.6-cm path length cell measured absorbance at 278 nm. The HPLC column in every case was eluted at room temperature (21 °C) at a flow rate of 1.5 mL/min. One-minute fractions were collected into borosilicate glass tubes (12 × 75 mm, Fisher Scientific) which had previously been rinsed with 80% acetonitrile containing 0.1% F_3CCOOH.[2] An LKB Ultravac 7000 fraction collector (Fisher Scientific, Montreal, Canada) was used throughout.

HPLC grade water was prepared from deionized, glass-distilled water which was passed slowly under gravity over a small bed of ODS-silica [the contents of a C_{18} Sep-Pak (Waters Associates) packed within a glass syringe] and stored in glass at 4 °C until used. Prior to the preparation of HPLC solvents, water was filtered with a Millipore all-glass filtration apparatus and 0.5-μm Fluoropore filters (Millipore Corp., Bedford, MA), which were wetted with acetonitrile before use. Stock solutions of 1% (v/v) F_3CCOOH (i.e., 0.13 M) and 1.3% (v/v) HFBA (i.e., 0.1 M) were prepared by using filtered water, and each was passed through an ODS-silica cartridge. Immediately before solvent preparation, water was degassed for 20 min and acetonitrile for 1–2 min. The stock acid solutions were diluted with water or acetonitrile and water during solvent preparation. Routinely, acetonitrile gradients were formed by using pump A (designated the "aqueous" pump) to deliver an aqueous solution of 0.08% or 0.1% F_3CCOOH or 0.13% HFBA and by using pump B (designated the "solvent" pump) to deliver 80% acetonitrile containing 0.08% or 0.1% F_3CCOOH or 0.13% HFBA. Samples were introduced into the chromatography system either via the injector, for the chromatography of peptide standards, or by pumping directly onto the HPLC column through one of the unused inlet parts of the aqueous pump.

Aliquots of column fractions for radioimmunoassay, enzyme digestion, or amino acid analysis were dried with a water aspirator at 37 °C and a National vacuum oven (Fisher Scientific, Montreal, Canada). Large volumes can be dried in the liquid state provided that they are previously chilled at about −10 °C for 30 min. This prevents the sample from "bumping" when the vacuum is applied. This method is preferable to freeze-drying because the sample clings to the surface of the container once the liquid phase has evaporated. The RP-HPLC solvents are completely volatile. No damage to ACTH or other peptides was apparent even when an overnight drying step was required.

Extraction and Purification of ACTH from Rat Anterior Pituitary Lobes. In a pilot experiment the stability and recovery of [^{3}H]ACTH through extraction and chromatography were determined. For this purpose ~25 ng of [^{3}H]ACTH

[2] The solvents used in this study were always simple mixtures of acetonitrile, water, and F_3CCOOH or HFBA made up by volume, e.g., an 80% acetonitrile solution containing 0.1% F_3CCOOH is a mixture of acetonitrile/water/F_3CCOOH in the ratio 800:199:1 (v/v).

(50000 cpm) was added to 10 whole rat pituitaries which were extracted with 2 mL of the homogenization medium. Further extraction and chromatography were performed as described below. Good recoveries of undamaged [^{3}H]ACTH (see Results) justified the following protocol.

Sixty male Sprague-Dawley rats (200–250 g, Canadian Breeding Farms, St. Constant, Canada) were sacrificed by decapitation, and each pituitary was removed and dissected into anterior and neurointermediary lobes. Within 1 min of death, the anterior lobes were homogenized at 4 °C with a ground-glass homogenizer in 5 mL of a medium consisting of 1 M hydrochloric acid, 5% formic acid (v/v), 1% sodium chloride (w/v), and 1% F_3CCOOH (v/v). After centrifugation, the pellet was rehomogenized with a second 5 mL of the extraction medium. The supernatants were combined and were passed 5 times through an ODS-silica cartridge (C_{18} Sep-Pak, Waters Associates). Before use, the ODS-silica was "wetted" with 5 mL of 80% acetonitrile containing 0.1% F_3CCOOH and then flushed with 5 mL of 0.1% F_3CCOOH to remove excess solvent. After loading, the cartridge was washed with 20 mL of 0.1% F_3CCOOH and eluted with 3 mL of 80% acetonitrile containing 0.1% F_3CCOOH. This fraction was diluted to 18 mL with 0.1% F_3CCOOH and was loaded directly onto the HPLC column through the aqueous pump at 3 mL/min. The column had previously been equilibrated with 12% acetonitrile containing 0.1% F_3CCOOH, and the sample was washed onto the column with a further 10 mL of this solution. The column was eluted with a linear gradient of 20–40% acetonitrile containing 0.1% F_3CCOOH throughout, for 1 h, and then purged for 10 min with 80% acetonitrile containing 0.1% F_3CCOOH. Fractions containing the main peak of immunoreactive ACTH were diluted 1:1 with 0.13% HFBA and pumped at a flow rate of 3 mL/min directly onto the same column which had been previously equilibrated with 12% acetonitrile containing 0.13% HFBA. The sample was washed onto the column with a further 10 mL of this solution. The column was eluted over 1 h with a linear gradient of 33.6–41.6% acetonitrile containing 0.13% HFBA throughout. In this system the main peak of immunoreactive ACTH from the first chromatography resolved into two components. To ensure complete purity for each form of ACTH, we diluted appropriate fractions 1:1 with 0.1% F_3CCOOH and loaded them onto the HPLC column (equilibrated and later washed with 10 mL of 12% acetonitrile containing 0.1% F_3CCOOH) at 3 mL/min. Each form of ACTH was chromatographed by using a linear gradient of 20–40% acetonitrile containing 0.1% F_3CCOOH throughout over 30 min. On this occasion each peak was collected by hand as it emerged from the column.

Each time an HPLC column is loaded via the aqueous pump, it is important to flush the loading solvent out of the pump head with 0.1% F_3CCOOH or 0.13% HFBA where appropriate. If this is not done, the initial solvent mixture generated by pumps A and B will be incorrect.

Extraction of Rat Plasma. Three male Sprague-Dawley rats (200–250 g) were anesthetized with ether and then exsanguinated by heart puncture. The blood was collected into heparinized plastic syringes. The plasma obtained after centrifugation at 4 °C was combined, and 3-mL portions were extracted by passage 10 times through ODS-silica cartridges. Peptide recoveries were assessed by the addition of [^{3}H]ACTH to plasma samples before ODS-silica extraction. Each cartridge was washed with 20 mL of 0.1% F_3CCOOH and eluted with 3 mL of 80% acetonitrile containing 0.1% F_3CCOOH. The extracts were diluted with 0.1% F_3CCOOH and pumped onto the column, and the column was eluted exactly as described for the pituitary extract.

Characterization of ACTH-like Peptides from the Rat Anterior Pituitary. (*a*) *Amino Acid Analysis.* Peptides were hydrolyzed in evacuated glass tubes in 0.25 mL of 6 M hydrochloric acid for 18 h at 115 °C. To prevent oxidation of methionine, we equilibrated the hydrochloric acid with nitrogen. Loss of tyrosine was prevented by the addition of a crystal of phenol to each hydrolysis. Amino acid analyses were performed with a Durrum Series D500 analyzer.

(*b*) *Trypsin Digestion.* Synthetic human ACTH (200 μg) and the two rat ACTH-like peptides (10 μg each) were digested with trypsin (diphenylcarbamoyl chloride treated, Sigma Chemical Co., St. Louis, MO). Digestion was carried out in 200 μL 0.05 of M ammonium bicarbonate (pH 8.5) at 37 °C for 14 h with an enzyme to peptide ratio of 1:100 (w/w). The digestions were quenched by the addition of 40 μL of 1% F_3CCOOH and injected into the HPLC system. In each case, the tryptic fragments were separated by RP-HPLC, using a linear gradient of 1.6–40% acetonitrile containing 0.1% F_3CCOOH over 1 h. Fragments of synthetic human ACTH were identified by amino acid analysis. Fragments obtained from the two forms of rat ACTH were identified by their elution positions compared to those obtained from synthetic human ACTH.

(*c*) *Polyacrylamide Gel Electrophoresis.* Sodium dodecyl sulfate–polyacrylamide gel electrophoresis [15% polyacrylamide, 0.4% *N*,*N*-methylenebis(acrylamide)] was performed as described by Swank & Munkers (1971). Peptide and protein bands were visualized by staining for 1 h in 0.25% Coomassie Brillant Blue in 50% methanol/9% acetic acid followed by destaining overnight in 7% acetic acid. For detection of peptides by radioimmunoassay (RIA), the gels were cut into 1-mm slices, and the peptides were eluted overnight at 4 °C in 2 mL of the RIA buffer (Browne et al., 1981). Aliquots of up to 200 μL of these samples could be assayed in all three of the RIA systems without any interference from $NaDodSO_4$.

(*d*) *ACTH Bioassay.* The corticotropic activity of peptides was determined by using an isolated rat adrenal cell suspension system as described by Sayers et al. (1971). Corticosterone production over a 2-h period was measured by a specific RIA, after extraction of the cell suspension with methylene chloride (10 volumes). The cell preparation responded to $ACTH_{1-39}$ and $ACTH_{1-24}$ but not to any other synthetic peptides tested including corticotropin-like intermediary-lobe peptide (CLIP), α-MSH, or α-, β-, and γ-endorphin. The potency of isolated peptides in this assay was always determined relative to synthetic human $ACTH_{1-39}$.

Radioimmunoassays. ACTH and α-MSH RIAs were performed as described in the following paper (Browne et al., 1981).

β-Endorphin RIA was performed by using a modification of the method of Guillemin et al. (1977), using a sample of RB100 antiserum kindly supplied by Dr. N. Ling, Salk Institute, CA. [^{125}I]Porcine β-endorphin was prepared by the chloramine-T method and was purified on a C_{18} Sep-Pak as described for ACTH (Browne et al., 1981). The assay was performed by using a final antiserum dilution of 1:140000 in an incubation volume of 0.7 mL for 18 h at 4 °C. The bound and free fractions were separated by the addition of 0.7 mL of a suspension of dextran-coated charcoal in buffer at 4 °C for 10 min. The working range of the assay was from 25 to 4000 pg. Under these assay conditions, the RB100 antiserum cross-reacted with human and ovine β-endorphin and ovine

Table I: Efficiency of Extraction of Tritiated $ACTH_{1-39}$ from Rat Pituitaries and Plasma[a]

	initial counts added	counts washed through ODS-silica cartridge	counts eluted from ODS-silica cartridge	total counts eluted from RP-HPLC column	counts eluted in main peak from RP-HPLC column
std [^{3}H]$ACTH_{1-39}$ (25 ng)	50 450[b] (100%)			50 200 (99%)	45 740 (91%)
[^{3}H]$ACTH_{1-39}$ (25 ng) in rat pituitary extract	42 250 (100%)	10 (<0.1%)	39 890 (94%)	44 510 (105%)	43 540 (103%)
high concn[d] of [^{3}H]$ACTH_{1-39}$ in rat plasma	50 450 (100%)	470 (0.9%)	46 270 (92%)	43 000 (85%)	42 210 (84%)
low concn[d] of [^{3}H]$ACTH_{1-39}$ in rat plasma	1 650 (100%)	nd[c]	nd[c]	1 350 (82%)	1 320 (80%)

[a] [^{3}H]ACTH was added to an homogenate of 10 rat pituitaries and 3 mL of rat plasma and extracted with ODS-silica cartridges. Recoveries of radioactivity were monitored throughout the ODS-silica extraction procedure and in the subsequent analysis of extracts by RP-HPLC. [b] Radioactivity expressed as counts per minute. [c] nd = not determined. [d] High and low concentrations of [^{3}H]$ACTH_{1-39}$ were 25 and 0.8 ng, respectively, per 3 mL of plasma.

β-LPH, but not with α-endorphin or γ-endorphin (<0.05%), in full agreement with the previously reported findings of Guillemin et al. (1977).

Radioactive Counting. Tritium was counted with a Beckman LS-7500 liquid scintillation counter employing automatic quench correction. Aliquots from tissue extracts and HPLC column fractions were counted in 5 mL of Scintiverse (Fisher Scientific, Montreal, Canada). [^{125}I]Iodine was counted with an LKB Wallac 1270 Rackgamma II γ counter.

Results

Extraction and Recovery of [^{3}H]ACTH from Tissue and Plasma. In order to demonstrate the efficiency of the extraction method, we added 25 ng of [^{3}H]ACTH (50000 cpm) to an homogenate of 10 rat pituitaries. Extraction with ODS-silica was followed by RP-HPLC, and recovery of radioactivity was monitored throughout the extraction and chromatographic procedures. Table I shows that the recovery of radioactivity for the complete process was close to 100%. After RP-HPLC almost all (98%) of the radioactivity eluted in one peak corresponding to the main peak of standard [^{3}H]$ACTH_{1-39}$ RP-HPLC of the standard ACTH (Figure 1C) revealed a small contaminating peak of radioactivity eluting three fractions early which is probably the methionine sulfoxide form of [^{3}H]$ACTH_{1-39}$. It has been reported that this product can be readily formed from ACTH by air oxidation and can be resolved from the unoxidized ACTH by RP-HPLC (Bennett et al., 1977). In order to demonstrate that our extraction procedures does not cause structural modification of ACTH, 20 μg of synthetic human ACTH was incubated in 5 mL of extraction medium for 20 h at 4 °C. Peptides were extracted with ODS-silica as described and analyzed by RP-HPLC, using both the gradient and isocratic elution conditions and employing both the F_3CCOOH and HFBA solvent systems. In every instance the ACTH coeluted with unextracted human ACTH.

Two experiments were performed to test the efficiency of the extraction of [^{3}H]$ACTH_{1-39}$ from plasma. In the first experiment, ~25 ng [^{3}H]ACTH (50000 cpm) was added to 3 mL of rat plasma which was subjected to ODS-silica extraction and RP-HPLC. The overall efficiency for this process was ~85% (Table I). After RP-HPLC, 99% of the counts eluted from the column corresponded to authentic [^{3}H]-$ACTH_{1-39}$ with only a small amount of the sulfoxide found (Figure 1B). In a second experiment ~0.8 ng of [^{3}H]-$ACTH_{1-39}$ (1700 cpm) was added to 3 mL of rat plasma and was similarly extracted and run on RP-HPLC. This lower amount is approximately equivalent to the physiological concentrations of $ACTH_{1-39}$ found in rat plasma. In this experiment, two of the intermediate steps in the extraction were not monitored since this would have resulted in a significant loss of radioactivity. The overall efficiency in this experiment

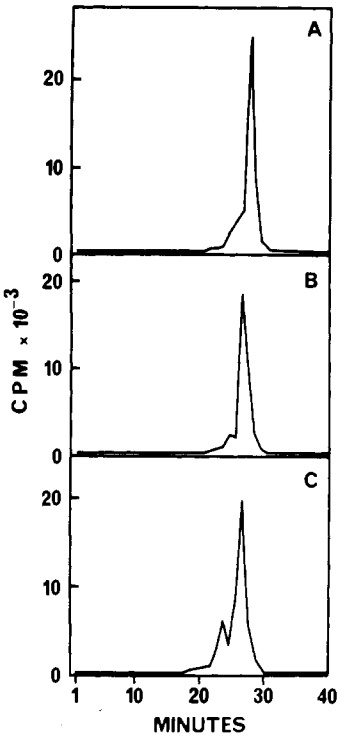

FIGURE 1: Reversed-phase HPLC of [^{3}H]$ACTH_{1-39}$ extracted from homogenate of rat pituitaries and plasma by ODS-silica cartridges. The RP-HPLC column was loaded and eluted as described under Experimental Procedures. Panel A is the radioactivity profile obtained from 10 rat pituitaries, panel B is the profile from 3 mL of rat plasma, and panel C is the profile of standard unextracted [^{3}H]$ACTH_{1-39}$.

was found to be ~80% (Table I), and again almost all (98%) of the radioactivity recovered after RP-HPLC eluted in a single peak corresponding to the elution position of the standard [^{3}H]$ACTH_{1-39}$ (data not shown).

Recovery of Endogenous Peptides from Pituitary Extracts. The efficiency of extraction and chromatography of endogenous peptides from pituitary tissue was assessed by using ACTH, α-MSH, and β-endorphin radioimmunoassays. Table II shows the cumulated information obtained from the three experiments. In each experiment the pituitaries from 60 rats were separated into anterior and neurointermediary lobes. Tissue was extracted and subjected to RP-HPLC exactly as described for the anterior lobes under Experimental Procedures. The results in Table II are expressed as micrograms of peptide per 60 lobes. In one experiment tissue was homogenized 3 times to test the efficiency of solubilization of peptides by the homogenization medium. Greater than 95% extraction was found for the first two homogenization steps for all three types of immunoreactivity.

Reversed-Phase Chromatography of Peptides. Chromatograms of seven standard peptides and proteins in two dif-

Table II: Extraction Efficiency of ACTH, α-MSH, and β-Endorphin Immunoreactive Peptides from Rat Pituitaries[a]

	μg of immunoreactive peptide/60 pituitaries			
	tissue extract	unretained by ODS-silica cartridge	ODS-silica cartridge eluate	total eluted from RP-HPLC column
anterior lobe				
ACTH	150 ± 7	5 ± 2	138 ± 28	168 ± 12
α-MSH	9 ± 1	1 ± 0.4	10 ± 1	nd[b]
β-endorphin	90 ± 9	5 ± 1	88 ± 6	77 ± 14
neurointermediary lobe				
ACTH[c]	152 ± 14	6 ± 2	136 ± 18	140 ± 15
α-MSH	144 ± 10	9 ± 1	148 ± 4	139 ± 10
β-endorphin	130 ± 11	5 ± 1	136 ± 11	122 ± 12

[a] In three separate experiments 60 rat pituitaries were dissected into anterior and neurointermediary lobes and subjected to ODS-silica extraction and RP-HPLC. Recoveries are given in microgram equivalents of immunoreactive peptides found per 60 pituitary lobes (±standard errors). [b] nd = not determined. [c] Most of this immunoreactive ACTH is corticotropin-like intermediary-lobe peptide (CLIP) (Browne et al., 1981).

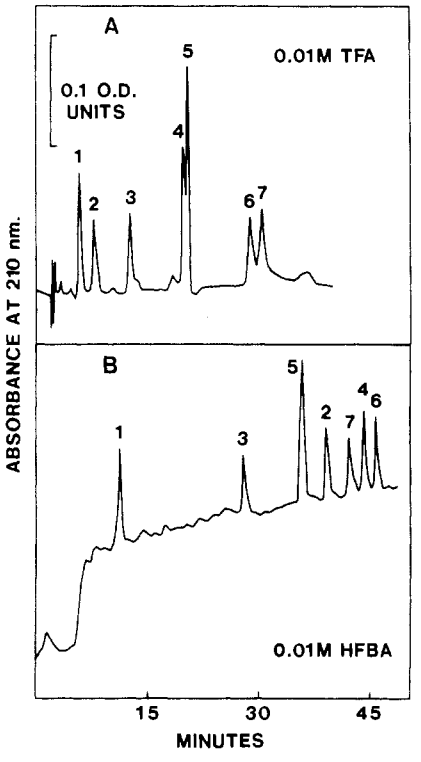

FIGURE 2: Comparison of effectiveness of trifluoroacetic acid (F_3C-COOH) and heptafluorobutyric acid (HFBA) as hydrophobic counter ions in reversed-phase HPLC of seven natural and synthetic peptides. The μBondapak column was eluted in both cases with a linear gradient of 20–40% aqueous acetonitrile containing 0.01 M concentrations of each acid over 1 h, at a flow rate of 1.5 mL/min. Panel A shows the elution behavior of the peptides with 0.01 M F_3CCOOH as counterion and panel B shows the corresponding results with 0.01 M HFBA. The peptides tested (2 μg each) were as follows: (1) methionine enkephalin; (2) Synacthen ($ACTH_{1-24}$); (3) α-MSH; (4) human $ACTH_{1-39}$; (5) somatostatin; (6) bovine insulin; (7) human calcitonin.

ferent RP-HPLC solvent systems are shown in Figure 2. In one of the chromatograms, the peptides have been separated by a gradient of aqueous acetonitrile containing 0.01 M F_3CCOOH throughout (Figure 2A) while in the second chromatogram, the solvent systems consisted of a gradient of aqueous acetonitrile containing 0.01 M HFBA (Figure 2B).

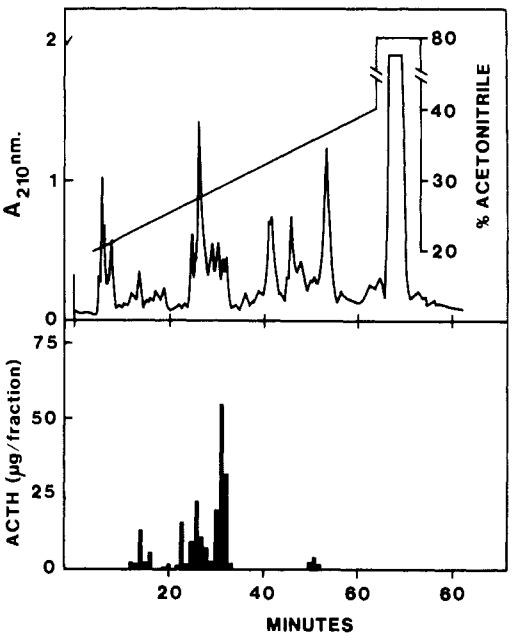

FIGURE 3: Reversed-phase HPLC of ODS-silica extract of 60 rat anterior pituitaries. The RP-HPLC column was eluted with a solvent system containing 0.1% F_3CCOOH as described under Experimental Procedures. The upper panel shows the UV absorbance at 210 nm. Immunoreactive ACTH, in micrograms per fraction, is shown as solid bars in the lower panel.

Several differences can be observed between the two chromatograms, the most important being that the order of elution of the peptide standards is different in the two chromatograms. Since the acetonitrile gradient is identical in both cases, it is clear that the behavior of these peptides is highly sensitive to the nature of the perfluorinated carboxylic acid. This effect can be taken advantage of in the isolation of peptides from biological sources. Crude homogenates of peptides are extracted by the ODS-silica method and are subjected to RP-HPLC with a solvent system containing F_3CCOOH. Any material of interest is reloaded onto the column and repurified by using a solvent system containing HFBA. This approach is demonstrated below in the isolation of ACTH from the anterior pituitary of the rat. Two other advantages of the above systems are that they are volatile and sufficiently transparent at 210 nm to facilitate UV monitoring at this wavelength.

Isolation of ACTH-Related Peptides from Rat Anterior Pituitary. The anterior lobes from 60 rat pituitaries were homogenized and extracted by using ODS-silica and subjected to RP-HPLC with a solvent system containing 0.1% F_3CCOOH (Figure 3). ACTH RIA indicated the presence of at least five different forms of immunoreactive ACTH. The main peak of ACTH immunoreactivity, which corresponded approximately to the elution position of standard human $ACTH_{1-39}$, was further purified by using a shallow acetonitrile gradient containing 0.13% HFBA (Figure 4). Examination of the column fractions for immunoreactivity revealed that the ACTH consisted of two components, which were now resolved from each other (peaks I and II) (Figure 4). Both immunoreactive peaks correspond to UV absorbance at 210 and 278 nm. The material which eluted first (peak I) appeared from both the UV and RIA profiles to be well resolved from any other components. The second compound (peak II), however, was clearly contaminated with some UV-absorbing material, which was eluted from the column slightly later than peak II. In order to purify the material in peak II completely and to establish the purity of peak I, we reran the materials in each

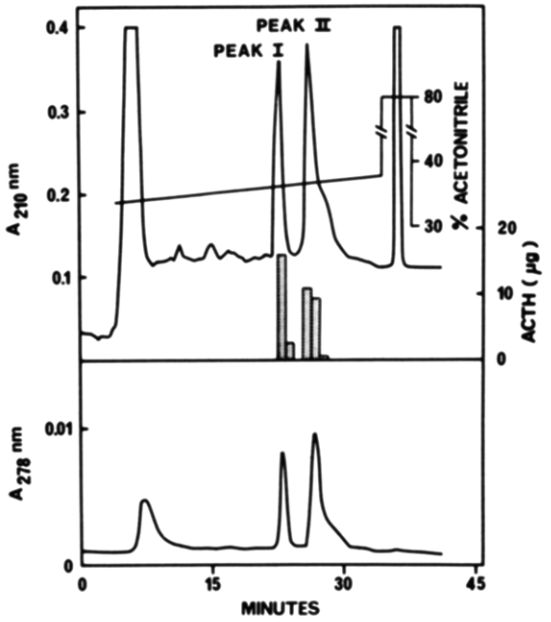

FIGURE 4: Reversed-phase HPLC of fractions 30, 31, and 32 from initial chromatography of extract of 60 rat anterior pituitaries (see Figure 3). The RP-HPLC column was eluted with a solvent system containing 0.13% HFBA as described under Experimental Procedures. In the upper panel the continuous line shows the UV absorbance at 210 nm, and the stippled bars show the ACTH immunoreactivity observed in micrograms per fraction. The lower panel shows the UV absorbance at 278 nm.

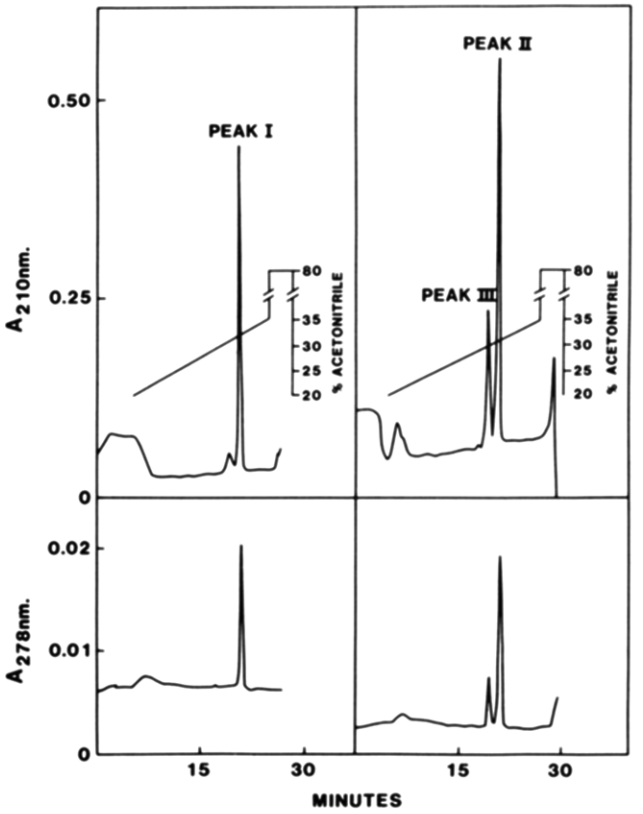

FIGURE 5: Reversed-phase HPLC of peaks I and II which were partially purified from extract of 60 rat pituitaries (see Figure 4). The RP-HPLC column was eluted with a linear gradient over 30 min from 20% to 40% acetonitrile containing 0.1% F_3CCOOH throughout as described under Experimental Procedures. The upper panels show the UV absorbance at 210 nm while the lower panels show the UV absorbance at 278 nm.

of these peaks separately by using chromatographic conditions similar to those used for the chromatogram in Figure 3 (i.e., a linear acetonitrile gradient over 30 min from 20% to 40% acetonitrile, containing 0.1% F_3CCOOH throughout). This procedure demonstrated that the material in peak I was pure (Figure 5) and that the material in peak II was now resolved from the contamination that had been previously apparent in Figure 4. The contaminating material was now eluted from the column as a sharp peak (peak III) just before peak II (Figure 5). Peaks I and II both consisted of immunoreactive ACTH which displaced the tracer in a manner parallel to that of human $ACTH_{1-39}$ in the ACTH RIA, and neither contained immunoreactive β-endorphin (data not shown). These two materials were now called ACTH I and ACTH II. Peak III was devoid of both ACTH and β-endorphin immunoreactivity.

Characterization of ACTH I and ACTH II. Half of each preparation (i.e., ~10 μg each of ACTH I and ACTH II) was acid hydrolyzed, and the products were subjected to amino acid analysis. The results (Table III) demonstrated that both of these peptides had analyses which corresponded very closely to that reported previously for rat $ACTH_{1-39}$ (Scott et al., 1974). The amino acid analysis data are of the precision expected for a sample of this size (i.e., ~2 nmol), which is close to the practical limit of sensitivity of the Durrum analyzer system as it is currently set up. For clarification the difference between these two forms of ACTH, samples of each of them were digested with trypsin, and the resultant tryptic peptides were separated by RP-HPLC (Figure 6, lower panel). Tryptic peptides were similarly prepared from synthetic human $ACTH_{1-39}$ (Figure 6 top panel), hydrolyzed, and subjected to amino acid analysis. Identity of the fragments of human ACTH could be deduced from their amino acid compositions. The peaks labeled TP3, TP5, and TP7 were found to correspond to expected ACTH tryptic peptides 9–15, 1–8, and 22–39, respectively. The four smaller peaks TP1, TP2, TP4, and TP6 correspond to fragments 18–21, 17–21, 3–8, and 22–37, respectively, of ACTH. Fragments 3–8 and 22–37 of both ACTH both result from chymotryptic cleavages of ACTH (Shepherd et al., 1956), presumably caused by contamination of the trypsin by residual chymotrypsin activity. Both ACTH I and II gave rise to tryptic fragments corresponding to 18–21, 17–21, 9–15, and 1–8 (i.e., TP1, TP2, TP3, and TP5) from human ACTH. The difference between ACTH I and ACTH II is confined to the carboxyl-terminal

Table III: Amino Acid Composition of the Two Major Forms of Corticotropin (ACTH) Isolated from 60 Rat Anterior Pituitaries

	ACTH I	ACTH II	rat ACTH [a]
Asx	2.0	2.0	2
Ser [b]	2.2	2.2	3
Glx	5.8	5.6	5
Pro	3.5	4.3	4
Gly	2.2	1.9	2
Ala	3.2	2.9	3
Val	4.5	4.0	4
Met	0.8	0.8	1
Leu	1.3	1.2	1
Tyr	2.2	2.0	2
Phe	2.8	2.7	3
His	1.1	0.9	1
Lys	3.7	3.9	4
Arg	3.0	3.3	3
Trp	nd [c]	nd [c]	1

[a] Amino acid composition for rat ACTH proposed by Scott et al. (1974). [b] Serine values have not been corrected for breakdown during acid hydrolysis. [c] nd = not determined because of destruction during acid hydrolysis, but the presence of one tryptophan residue in each ACTH is indicated by the ratio of the UV absorbance at 278 nm to that at 210 nm (Figure 4) when compared to the ratio obtained for synthetic $ACTH_{1-39}$.

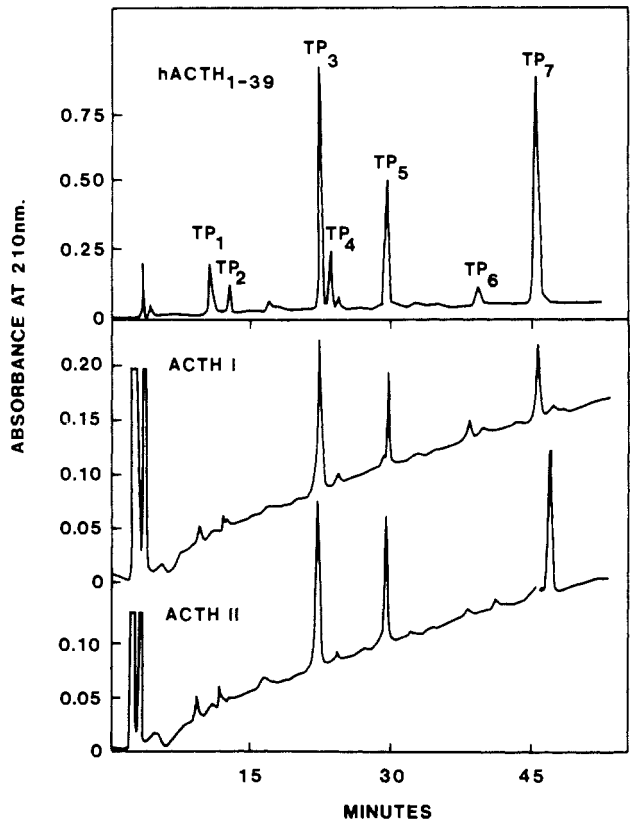

FIGURE 6: Tryptic fragments of synthetic human $ACTH_{1-39}$ (upper panel) and isolated rat ACTH I and II (lower panel) separated by reversed-phase HPLC (see Experimental Procedures for details). The F_3CCOOH solvent system was used because it gives a more stable UV absorbance profile than the solvent system containing HFBA.

fragment corresponding to TP7 (i.e., 22–39). It follows that the difference between ACTH I and ACTH II cannot be attributed to oxidation, since the amino acid susceptible to oxidation (i.e., the methionyl residue at position 4) is found in the tryptic fragment corresponding to TP5.

The molecular weights for ACTH I and ACTH II were determined by $NaDodSO_4$–polyacrylamide gel electrophoresis. Apparent molecular weights of 5800 ± 200 were obtained for both ACTH I and ACTH II. Synthetic human $ACTH_{1-39}$ gave an apparent molecular weight of 5900 ± 300 in the same $NaDodSO_4$–polyacrylamide gel electrophoresis system. Although these apparent molecular weights differ from the true molecular weight of $ACTH_{1-39}$ (≈4500), they do indicate that ACTH I, ACTH II, and synthetic $ACTH_{1-39}$ have similar molecular weights. ACTH I and ACTH II showed only one band on the gel electrophoresis system, both by staining with Coomassie Blue and by RIA. Samples of ACTH I and ACTH II which were mixed and run on the same gel were not resolved from one another. When ACTH I and ACTH II were tested for biological activity, they were found to be apparently equipotent with synthetic human $ACTH_{1-39}$ in the isolated adrenal cell bioassay.

Discussion

We have demonstrated in this paper the potential of a novel procedure for the extraction and isolation of peptides from tissues and body fluids. The extraction of peptides from tissues by homogenization in an acid medium followed by extraction of the homogenate supernatant with ODS-silica is a highly efficient process for the extraction of ACTH-, α-MSH-, and β-endorphin-related peptides (Tables I and II). The control experiments with synthetic tritiated ACTH and unlabeled human ACTH demonstrated that the integrity of the molecule was fully preserved through the extraction and chromatography procedures. There was no evidence of any artifactual alteration of the peptide. The extraction process is a modification of a method described previously (Bennett et al., 1978). The high concentration (15% v/v) of F_3CCOOH used in the original procedure has been reduced to 1% in order to permit extraction of larger peptides, which would be precipitated by 15% F_3CCOOH.

The ODS-silica used in this study for the extraction of peptides from plasma and tissue homogenates was contained in syringe-fitting cartridges (C_{18} Sep-Pak). These cartridges are more convenient and reproducible than ODS-silica packed in disposable syringes, as described in the original method. Sep-Pak cartridges are also radially compressed during manufacture making the interaction of aqueous extracts and plasma with the hydrophobic surface more efficient than an open column system. It has become clear recently that pore size (300 Å for ODS-Porasil C, the packing material in Sep-Pak cartridges) is an important factor in determining the capacity of ODS-silica for peptides and proteins (Lewis et al., 1980; van der Rest et al., 1980). Small peptides tend to penetrate pores of ODS-silica more efficiently than proteins. Indeed some proteins may be excluded altogether. This effect is illustrated by considering that, although 3 mL of plasma contains ~200 mg of protein (Tietz, 1970), small quantities of [^{3}H]ACTH are completely extracted by a single ODS-silica cartridge. Overloading of ODS-silica cartridges occurs with ~4 mL of plasma or with extractions of large amounts of tissue. This problem can be circumvented by using several ODS-silica cartridges to extract peptides from large-scale homogenates or large volumes of protein-rich biological fluids. Since fats also overload ODS-silica, a solvent extraction procedure is required in some large-scale applications of this methodology (Brubaker et al., 1980).

Several workers have demonstrated that peptides can be readily separated by RP-HPLC. The most successful methods all depend on the presence of an ionic modifier or "ion-pairing" reagent in the solvent system (Hearn & Hancock, 1979). The modifiers used fall into two broad groups: acids at pH 2 to 3 such as trifluoroacetic acid (Bennett et al., 1977), phosphoric acid (Hancock et al., 1978), and hydrochloric acid (O'Hare & Nice, 1979) or buffered salts such as triethylammonium phosphate (Rivier, 1978) or ammonium acetate (Rivier et al., 1977). Previous work from this laboratory has demonstrated that it is feasible to purify ACTH-related peptides from pituitary extracts to homogeneity by the use of RP-HPLC alone (Bennett et al., 1980a,b). In these studies, the approach was to chromatograph the initial extract by using a gradient of aqueous acetonitrile containing 0.1% F_3CCOOH, followed by rechromatography of any material of interest under the appropriate isocratic conditions. This approach has several drawbacks. First, a very similar type of chromatography was employed in both steps, and secondly in the isocratic chromatography it was difficult to select the appropriate conditions with sufficient certainty of success. The peptide of interest also tended to be eluted from the column in a relatively large volume of solvent as a rather broad peak. In order to rectify these problems, we investigated several alternatives to F_3CCOOH (Bennett et al., 1980c). The most promising one was heptafluorobutyric acid (HFBA). Chromatograms of several peptide standards, performed by using either F_3CCOOH (Figure 2A) or HFBA (Figure 2B) as counter ion, demonstrated that very good separations could be achieved with the use of these two perfluorinated carboxylic acids. Furthermore, both the order of elution and the retention times are clearly

different in the two chromatograms. The retention times of all of the peptides are greater in the HFBA chromatogram. The peptides with the greatest number of basic groups at pH 2 ($ACTH_{1-24}$ and $ACTH_{1-39}$, peaks 2 and 4, respectively, in Figure 2) show large increases in retention times, when comparing the HFBA system to the F_3CCOOH system. In contrast, peptides such as calcitonin (peak 7) or methionine enkephalin (peak 1), which contain relatively few basic groups, show smaller increases. Furthermore, some peptides such as $ACTH_{1-39}$ and somatostatin (peaks 4 and 5, respectively), which are only partially resolved by using F_3CCOOH (Figure 2B), are completely resolved by using HFBA (Figure 2A). The same situation could equally well apply in reverse, in that two or more peptides, which might only be poorly or partially resolved in the HFBA system, might be completely resolved in the F_3CCOOH system. These effects can be used to great advantage in the isolation and purification of peptides from biological sources.

To illustrate this principle, we have isolated two major forms of ACTH from the rat anterior pituitary by the use of the reversed-phase methodology alone. The first F_3CCOOH system (Figure 3) provides an efficient, initial separation of the complex mixture, derived from the ODS-silica extract. The HFBA system (Figure 4) has resolved ACTH I from ACTH II and peak III while the second F_3CCOOH system has resolved ACTH II from peak III. This three-step procedure permitted the rapid isolation of ACTH I and ACTH II in highly purified forms. Both ACTH I and ACTH II were isolated in high yield as is apparent from the heights of their UV peaks at 210 nm in Figures 3–5. The amino acid analyses (Table III) indicate the similarity of ACTH I and II. The tryptic peptide "maps" (Figure 6) indicated the purity of these two forms of ACTH and demonstrated that the difference between them is located in the carboxyl-terminal tryptic fragment $ACTH_{22-39}$. Neither ACTH I nor ACTH II bound to a Con A–Sepharose affinity column (unpublished observations), suggesting that they are not glycosylated forms of ACTH. These two forms of ACTH are very similar in biological activity to synthetic human $ACTH_{1-39}$ and gave the same apparent molecular weight as synthetic human $ACTH_{1-39}$ on $NaDodSO_4$–polyacrylamide gel electrophoresis. The finding of these two forms indicates that a microheterogeneity exists in the familiar 1–39 form of ACTH. In this present study, insufficient amounts of these two forms of ACTH were available for further characterization. We have recently isolated and identified two forms of corticotropin-like intermediary-lobe peptide (CLIP) from the rat neurointermediary pituitary, of which the more polar form of CLIP has an *O*-phosphoserine residue at position 31 whereas the less polar form has underivatized serine (Browne et al., 1981). Thus, a tentative explanation for the two forms of ACTH would then be that ACTH II has a serine residue at position 31 and that ACTH I has an *O*-phosphoserine residue at that position. Preliminary data obtained from the in vitro ^{32}P labeling experiments have demonstrated that ACTH I does contain *O*-phosphoserine at position 31 and that ACTH II does not (Bennett et al., 1981).

Peptides are extremely susceptible to enzyme attack, and the risk is high of purifying a peptide which has been generated during isolation. The best documented example of this hazard is the isolation of human β-MSH (Harris, 1959). This peptide has subsequently been found to be an extraction artifact (Scott & Lowry, 1974; Barat et al., 1979). This illustrates the importance of inhibiting peptidase activity during peptide isolation. We have found that the only consistent way of completely halting peptidase activity is to employ an extraction medium with a very low pH (i.e., 1 M HCl) at 4 °C. We have found no evidence for chemical peptide modification (i.e., deamidation or oxidation) in our isolations. We believe that the methodology described here represents a new general approach for the extraction and purification of peptides, which has several advantages over more traditional methods. It is simple, efficient, and rapid and requires only a single type of chromatographic equipment and expertise. Recoveries of unmodified peptides are optimized by using the ODS-silica extraction procedure and by minimizing chromatographic manipulations. High yields are also ensured by direct reloading of HPLC columns between each chromatographic step and by eliminating solvent evaporation until homogeneity has been achieved. In our current investigations, we have used the reversed-phase method of extraction and chromatography to isolate a variety of peptides of high purity, which we have successfully identified. It is, however, possible that situations may arise where there is a need to combine other chromatographic methods with those described above in order to obtain a homogeneous product. The ACTH-related peptides which have been isolated by this method retain full biological activity (Baird et al., 1980; Brubaker et al., 1980), as does human parathyroid hormone which has been similarly isolated by this methodology (Bennett et al., 1980d). The method can be conveniently scaled up by changing the initial extraction step from one Sep-Pak to several Sep-Paks for milligram quantities of peptides. In our laboratory, 6 mg of ACTH has been isolated from an ODS-silica extract of 40 bovine anterior pituitaries (32 g wet weight) by the use of an analytical RP-HPLC column (Brubaker et al., 1980). For gram quantities of peptides, a larger open column of ODS-silica and perhaps a larger RP-HPLC column might be required, but the same principles of chromatography can be applied.

Acknowledgments

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Isolation and Characterization of Corticotropin- and Melanotropin-Related Peptides from the Neurointermediary Lobe of the Rat Pituitary by Reversed-Phase Liquid Chromatography†

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ABSTRACT: A novel procedure utilizing reversed-phase high-performance liquid chromatography for the extraction and purification of peptides from biological tissues has been applied to the isolation of corticotropin-like intermediary lobe peptide (CLIP) and α-melanocyte-stimulating hormone (α-MSH) from the neurointermediary lobe of the rat pituitary. The isolation and characterization of two major forms of CLIP and two major forms of α-MSH are described. The isolated peptides have been identified by using enzymatic digestions and peptide mapping. The main form of CLIP is a peptide which has been modified by phosphorylation of the serine residue at position 31. This is the first peptide of endocrine origin reported to be modified in such a manner. A non-phosphorylated form of CLIP was also present at lower concentrations. The main form of α-MSH was found to be *N*,*O*-diacetyl-α-MSH, with the more familiar mono-*N*-acetyl-α-MSH present to a much smaller extent. Thus, in the rat neurointermediary lobe, the two main corticotropin-related peptides present are mostly in modified forms which are the result of posttranslational modifications. It is only by the use of methodology such as that described in this paper that small alterations in peptide structure may be identified.

It has been known for many years that the intermediary lobe of the pituitary contains large quantities of various peptide hormones. α-Melanotropin (α-MSH)[1] has been isolated from the rat intermediary lobe (Harris, 1956), and more recently corticotropin-like intermediary lobe peptide (CLIP;

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[1] Abbreviations used: RP-HPLC, reversed-phase high-performance liquid chromatography; F_3CCOOH, trifluoroacetic acid; HFBA, heptafluorobutyric acid; ODS-silica, octadecylsilylsilica; α-MSH, α-melanocyte-stimulating hormone; ACTH, adrenocorticotropic hormone; CLIP, corticotropin-like intermediary-lobe peptide; β-LPH, β-lipotropic hormone; RIA, radioimmunoassay; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/81/0420-4538$01.25/0